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# Influence of size and polarity of residue 31 in porcine pancreatic phospholipase A<sub>2</sub> on catalytic properties

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**Residue 31 of porcine pancreatic phospholipase A<sub>2</sub> (PLA<sub>2</sub>) is located at the entrance to the active site. To study the role of residue 31 in PLA<sub>2</sub>, six mutant enzymes were produced by site-directed mutagenesis, replacing Leu by either Trp, Arg, Ala, Thr, Ser or Gly. Direct binding studies indicated a three to six times greater affinity of the Trp31 PLA<sub>2</sub> for both monomeric and micellar substrate analogs, relative to the wild-type enzyme. The other five mutants possess an unchanged affinity for monomers of the product analog *n*-decylphosphocholine and for micelles of the diacyl substrate analog *rac*-1,2-dioctanoylamino-dideoxy-glycero-3-phosphocholine. The affinities for micelles of the monoacyl product analog *n*-hexadecylphosphocholine were decreased 9–20 times for these five mutants. Kinetic studies with monomeric substrates showed that the mutants have  $V_{\max}$  values which range between 15 and 70% relative to the wild-type enzyme. The  $V_{\max}$  values for micelles of the zwitterionic substrate 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine were lowered 3–50 times. The  $K_m$  values for the monomeric substrate and the  $K_m$  values for the micellar substrate were hardly affected in the case of five of the six mutants, but were considerably decreased when Trp was present at position 31. The results of these investigations point to a versatile role for the residue at position 31: involvement in the binding and orientating of monomeric substrate (analog), involvement in the binding of the enzyme to micellar substrate analogs and possibly involvement in shielding the active site from excess water. **Key words:** phospholipase A<sub>2</sub>/site-directed mutagenesis/lipolysis**

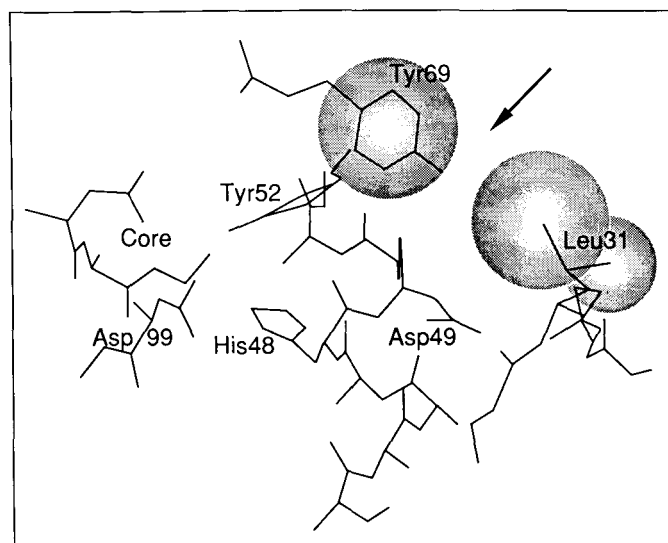
## Introduction

The lipolytic enzyme phospholipase A<sub>2</sub> (EC 3.1.1.4) specifically hydrolyzes the 2-acyl linkage of phosphoglycerides in a calcium-dependent reaction (Waite, 1987). Phospholipases occur both extracellularly and intracellularly. The extracellular enzymes are found abundantly in mammalian pancreas and in snake or bee venoms serving a digestive function. The intracellular phospholipases, which are thought to play an important role in inflammation processes, are found in low concentrations in nearly every mammalian cell (Waite, 1987). A high degree of sequence homology is found in phospholipases of over 50 species (van den Bergh *et al.*, 1989). All these PLA<sub>2</sub>s have a molecular mass between 13.5 and 14 kd and contain six or seven disulfide bridges. The three-dimensional (3-D) structures of several PLA<sub>2</sub>s from bovine and porcine pancreas and from *Crotalus atrox* venom have been determined by X-ray crystallography and

demonstrate that these enzymes are structurally similar (Dijkstra *et al.*, 1981a, 1983; Renetseder *et al.*, 1985; Brunie *et al.*, 1985).

An intriguing property of lipolytic enzymes is their ability to hydrolyze aggregated substrates, e.g. micelles, vesicles and liposomes, at much higher velocities than monomeric substrate molecules. The hydrolysis of aggregated substrates first requires the binding of the enzyme to the lipid–water interface. After binding of the enzyme to the lipid aggregate, one single substrate molecule is thought to diffuse into the active site, where it is hydrolyzed. A mechanism for catalysis has been proposed previously (Verheij *et al.*, 1980).

Chemical modification studies and semisynthesis of phospholipases from various sources have indicated Leu2, Trp3, Arg6, Leu19, Met20, Leu31 and Tyr69 in the porcine enzyme to be involved in the binding of the enzyme to aggregated phospholipids (Volwerk and de Haas, 1982). Inspection of the three-dimensional structures of bovine and porcine pancreatic PLA<sub>2</sub>s shows that these residues are present as a cluster of mainly hydrophobic and positively charged residues, located at one face of the enzyme, and surrounding the active site (Dijkstra *et al.*, 1981b). The three-dimensional structures of the pancreatic enzymes show that Leu31 and Tyr69 both lie at the entrance to the active site (Dijkstra *et al.*, 1981b). The position of these residues with respect to the active site is illustrated in Figure 1. Recently, Tyr69 has been the subject of a site-directed mutagenesis study, showing the involvement of this residue in the binding of the phosphate moiety of phospholipids (Kuipers *et al.*, 1989a). A comparison of the sequences of phospholipases



**Fig. 1.** Cross section of the active site of phospholipase A<sub>2</sub>. Section through the middle of the enzyme showing the essential residues for catalysis (His48, Asp49 and the C<sub>α</sub> of Asp99). The van der Waals surfaces of Leu31 and Tyr69, at the entrance of the active site, are shaded. Substrate molecules are supposed to approach the cleft from the direction of the arrow.

	25	30	35	40	45	50																						
1.	Y	G	C	Y	C	G	L	G	G	S	G	T	P	V	D	E	L	D	R	C	C	E	T	H	D	N	C	Y
2.	*	*	*	*	*	*	R	*	*	*	*	*	*	*	*	D	*	*	*	*	*	Q	I	*	*	*	*	*
3.	*	*	*	*	*	*	A	*	*	*	*	*	*	*	*	*	*	*	*	*	*	K	I	*	*	*	*	*
4.	*	*	*	*	*	*	W	*	*	K	*	K	*	I	*	A	T	*	*	*	*	F	V	*	*	C	*	*
5.	*	*	*	*	*	*	S	*	*	R	*	K	*	K	*	A	T	*	*	*	*	F	V	*	*	C	*	*
6.	*	*	*	*	*	*	G	*	*	Q	*	K	*	K	*	G	T	*	*	*	*	F	V	*	*	C	*	*

**Fig. 2.** Comparison of the sequences of porcine pancreatic and five snake venom phospholipases  $A_2$  in the region of residue 31. 1, Porcine pancreatic; 2, *Naja melanoleuca* fraction DE III; 3, *Laticauda semifasciata* fraction I; 4, *Bitis gabonica*; references to the publication of these (and many other) sequences have been published by Dufton *et al.* (1983). 5, *Agkistrodon halys blomhofii* basic (Forst *et al.*, 1986); 6, *Agkistrodon halys* Pallas (Kondo *et al.*, 1989). Sequence numbering is according to Renetseder *et al.* (1985).

from different species shows that in mammalian pancreatic phospholipases a Leu is invariably present at position 31, whereas in >85% of the known snake venom  $PLA_2$  sequences Trp, Arg or Ala is found (van den Bergh *et al.*, 1989). A comparison of the porcine pancreatic sequence with those of five representative snake venom  $PLA_2$ s in the region of residue 31 is given in Figure 2. Chemical modification of Trp31 in *Bitis gabonica* by *N*-bromosuccinimide greatly diminished enzymatic activity (Viljoen *et al.*, 1976). The inability of the modified enzyme to bind to the aggregated substrate was given as an explanation for this decrease in activity.

To study the role of residue 31 in more detail, six amino acid substitutions for Leu at position 31 of porcine pancreatic phospholipase  $A_2$  were introduced by site-directed mutagenesis. A degenerated oligonucleotide, yielding simultaneously the mutations Trp, Arg, Ala, Thr, Ser and Gly at position 31, was used for this purpose. The effects of the substitutions on the catalytic activities of the mutant enzymes on both monomeric and micellar substrates were determined. The binding properties of the mutants to monomeric and micellar substrate analogs were investigated with fluorescence spectroscopy.

## Materials and methods

### Construction and purification of mutant phospholipases

*Escherichia coli* K-12 strain PC 2494 [ $\Delta$ (lac-pro), sup E, thi/F' tra D<sub>36</sub>, pro A<sup>+</sup>B<sup>+</sup>, lac I<sup>q</sup>, lacZ  $\Delta$ M15; Phabagen collection, Utrecht] was used for plasmid constructions and as a host for M13-derived vectors. HB 2154 [ara,  $\Delta$  (lac-pro), thi/F' pro A<sup>+</sup>B<sup>+</sup>, lac I<sup>q</sup>, lacZ  $\Delta$ M25, mut L::Tn10] (Carter *et al.*, 1985) was used as recipient strain in the mutagenesis experiments. Substitutions in the pro $PLA_2$ -cDNA (de Geus *et al.*, 1987) were introduced by the gapped duplex procedure, using amber selection (Kramer *et al.*, 1984). The degenerated mutagenic oligonucleotide 5'-C.TGA.TCC.ACC.C(G or C)(A or T or C).GCC.ACA.GTA.GC-3' was used for site-directed mutagenesis at position 31. It was synthesized on a Bioscience 6800 DNA synthesizer. The sites of mutation in the sequence of the oligonucleotide are underlined. The resulting mutant pro $PLA_2$ -cDNA was sequenced by the dideoxy-chain-termination method after plaque purifying (Sanger *et al.*, 1977) and a *Bam*HI–*Bst*XI fragment of each mutant pro $PLA_2$ -cDNA was cloned into the expression vector described by Kuipers *et al.* (1989a). After transformation and expression in *E. coli* K-12 strain MC4100 (Casadaban, 1976), containing plasmid pCI857, the

mutant phospholipases were obtained by tryptic cleavage of reoxidized fusion protein (de Geus *et al.*, 1987) and purified by CM-cellulose chromatography at pH 5 and 6. Final purification to homogeneity was achieved on DEAE-cellulose at pH 8.0. Protein concentrations were determined from the absorbance at 280 nm, using an  $E_{1\text{cm}}^{1\%} = 13.0$  for the wild-type and five mutant enzymes, and an  $E_{1\text{cm}}^{1\%} = 18.0$  for the Trp31 mutant. Stock solutions of  $PLA_2$  routinely contained at least 0.25 mg/ml.

### Phospholipids

The 1,2-diacyl-*sn*-glycero-3-phosphocholines used in this study were obtained after reacylation of *sn*-glycero-3-phosphocholine. *Rac*-1,2-dihexanoyldithio-glycero-3-phosphocholine (diC6dithioPC) was obtained as described by Volwerk *et al.* (1979). *N*-Decylphosphocholine (C10PN) and *n*-hexadecylphosphocholine (C16PN) were prepared as described by van Dam-Mieras *et al.* (1975). The diacyl phospholipid analog *rac*-1,2-dioctanoylamino-dideoxy-glycero-3-phosphocholine (diC8diamidoPC) was prepared in analogy to the methods described by Bensen *et al.* (1972) and by Dijkman *et al.* (1990).

### Phospholipase assays

Quantitative measurements with 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine (diC8PC) as a substrate were carried out with a titrimetric assay at pH 8 in the presence of 1 mM borate, 25 mM CaCl<sub>2</sub> and 100 mM NaCl at 25°C. The burette of the Radiometer ABU was filled with 10 mM sodium hydroxide. Activities on monomeric diC6dithioPC were determined at pH 8 in the presence of 200 mM Tris, 100 mM NaCl and 100 mM CaCl<sub>2</sub>, as described previously (Volwerk *et al.*, 1979).

### Direct binding of $PLA_2$ to monomers and micelles

The affinity of phospholipase  $A_2$  for monomers and micelles was determined by following the increase of tyrosine and tryptophan fluorescence upon addition of increasing concentrations of the non-hydrolysable substrate analogs C10PN (CMC 10 mM) or diC8diamidoPC (CMC 1.7 mM) for monomer binding, and C16PN (CMC 10  $\mu$ M) or diC8diamidoPC for micelle binding. Fluorescence studies were performed in a buffer containing 100 mM NaAc, 50 mM CaCl<sub>2</sub> and 100 mM NaCl at pH 6.0. Excitation wavelength was at 280 nm. From saturation curves, obtained with lipid monomers, a  $K_d$  value can be derived directly. The data concerning micelle binding were analyzed in terms of the binding of the enzyme to a theoretical lipid particle consisting of *N* monomers with a dissociation

**Table I.** Binding of wild-type and six mutant PLA<sub>2</sub>s to the monomeric substrate analogs C10PN and diC8diamidoPC and kinetic constants measured with the monomeric substrate diC6dithioPC

Enzyme	C10PN	diC8diamidoPC	diC6dithioPC		
	$K_d$ (mM)	$K_d$ (mM)	$K_m$ (mM)	$k_{cat}$ (s <sup>-1</sup> )	$k_{cat}/K_m$ (s <sup>-1</sup> /M)
Wild-type (Leu31)	3.3	0.8	0.8	0.71	900
Trp31	0.6	0.3	0.2	0.52	2450
Arg31	3.0	0.9	0.7	0.26	380
Ala31	3.0	1.0	0.7	0.12	160
Thr31	3.1	1.0	1.0	0.11	110
Ser31	2.9	0.8	1.0	0.16	160
Gly31	4.1	0.8	1.0	0.07	70

C10PN stands for *n*-decylphosphocholine, diC8diamidoPC stands for *rac*-1,2-dioctanoylamino-dideoxy-glycero-3-phosphocholine and diC6dithioPC stands for *rac* 1,2-dihexanoyldithio-*sn*-glycero-3-phosphocholine. Standard errors were ~10% of each given value. For details see Materials and methods.

**Table II.** Binding of wild-type and six mutant PLA<sub>2</sub>s to the micellar substrate analogs C16PN and diC8diamidoPC and enzymatic activities on two micellar substrates

Enzyme	C16PN	diC8diamidoPC	diC8PC	$V_{max}$ ( $\mu$ mol/min/mg)
	$N \cdot K_d$ ( $\mu$ M)	$N \cdot K_d$ (mM)	$K_m$ (mM)	
Wild-type (Leu31)	70	3.9	3.7	2000
Trp31	17	0.8	0.5	600
Arg31	640	3.5	3.7	180
Ala31	570	3.2	3.9	60
Thr31	>1200	3.7	3.3	50
Ser31	>1200	4.3	3.7	50
Gly31	>1200	4.6	5.9	40

The substrate concentration of 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine (diC8PC) was varied between 0.5 and 10 mM. C16PN stands for *n*-hexadecylphosphocholine and diC8diamidoPC stands for *rac*-1,2-dioctanoylamino-dideoxy-glycero-3-phosphocholine. Accuracy was ~10% for each given value. For details see Materials and methods.

constant  $K_d$ . As has been discussed extensively by de Araujo *et al.* (1979), the  $N \cdot K_d$  value is the experimental concentration at which 50% of the enzyme is saturated with micelles.

## Results

### *Binding properties of wild-type and mutant PLA<sub>2</sub>s to monomeric substrate analogs and enzymatic activities on monomeric substrates*

The binding properties of wild-type and six PLA<sub>2</sub>s mutated at residue 31 were determined by fluorescence spectroscopy, using C10PN and diC8diamidoPC as substrate analogs at concentrations below the critical micelle concentration (CMC). Kinetic studies with these enzymes were performed with the monomeric substrate diC6dithioPC. The results of these studies are summarized in Table I. The dissociation constants for monomeric C10PN and diC8diamidoPC did not change significantly for most mutant enzymes, with the exception of the smallest residue at position 31, i.e Gly, which caused a small loss of affinity for monomeric C10PN. This fact might be explained by a slightly changed loop structure, in view of the general notion that a Gly residue can locally increase the flexibility of a peptide chain. A significant increase in affinity was observed only when a Trp was present. The  $K_m$  values determined with the monomeric substrate diC6dithioPC show the same tendency as the  $K_d$  values obtained with the direct binding studies. The fact, however, that most mutations at position 31 in PLA<sub>2</sub> hardly affected the dissociation constants for C10PN and diC8diamidoPC monomers, nor the  $K_m$  value for diC6dithioPC monomers, indicates that the side chain of residue 31 is not directly involved in the binding of the

monomeric substrate molecule. Apparently, only the Trp side chain is bulky and hydrophobic enough to give a substantial contribution to the binding of monomeric phospholipids via hydrophobic interaction with one or with both of the acyl chains of the substrate analog molecule.

Although the kinetic studies show a minor effect of the mutations on the affinity for monomeric substrates except in the case of Trp, there is considerable effect on the  $k_{cat}$  value. The effects on  $k_{cat}$  could be related to the size and/or polarity of the residue at position 31. An increased polarity (Arg) or a decreased size (Ala, Thr, Ser, Gly) cause a decrease in  $k_{cat}$ . It is, however, hard to predict the exact effect of size and polarity of residue 31 on  $k_{cat}$ , as long as a detailed understanding of substrate and transition state binding is not available. The mutant enzymes, except the Trp31 mutant, all have a significantly lower specificity constant ( $k_{cat}/K_m$ ) than the wild-type enzyme, mainly due to the reduced  $k_{cat}$  values.

### *Binding properties of wild-type and mutant PLA<sub>2</sub>s to micellar substrate analogs and enzymatic activities on micellar substrates*

The binding properties of wild-type and six mutant enzymes to micelles were determined by fluorescence spectroscopy, with the monoalkyl substrate analog C16PN and the diacyl substrate analog diC8diamidoPC (Table II). Because the former substrate analog contains only one alkyl chain, whereas the latter one contains two acyl chains, the mode of binding of these molecules by PLA<sub>2</sub> could be different. This was indeed observed, since from the data in Table II it is clear that the mutants have a decreased affinity for C16PN micelles, but retain their affinity for micelles of diC8diamidoPC. Only the Trp31 mutant displayed

an increased affinity for micelles of both C16PN and diC8diamidoPC.

Kinetic studies with micellar zwitterionic diC8PC were performed titrimetrically using a pH stat (Table II). With micellar substrates saturation curves are obtained which can be interpreted as Michaelis–Menten type of kinetics yielding  $V_{\max}$  and  $K_m$  values. However, as has been discussed extensively by Verger and de Haas (1976), the kinetic constants are apparent values. This is caused by the fact that the enzyme binds to its substrate in a two-step process. First, interaction occurs between the enzyme and the micelle, and subsequently a single substrate molecule is bound in the active site where hydrolysis occurs. By adding more micelles all the enzyme can become bound to the interface, but the concentration of monomers in the interface, and consequently the active site occupancy, remains constant. The kinetic analyses with this substrate at concentrations well above the CMC, revealed that for all mutants the  $K_m$  value had not changed greatly, except in the case of the Trp31 mutant. A similar tendency can also be observed when looking at the experimental values at which half of the enzyme is saturated with lipids ( $N \cdot K_d$  values) as they are obtained in the direct binding studies with the substrate analog diC8diamidoPC. In contrast to the minor effects of the mutations on the  $K_m$  values, the mutations have a large effect on the observed  $V_{\max}$  values. In the case of the Trp31 mutant this effect is only moderate, but the introduction of more polar and/or smaller amino acid side chains gives rise to 10–50 times lower  $V_{\max}$  values.

## Discussion

When the affinity of (mutant) phospholipases for monomeric substrate analogs was determined, no divergence in the relative affinities of these enzymes for substrate analogs containing either one alkyl or two acyl chains was observed. Thus, both types of analog seem to be appropriate molecules for studying the binding of PLA<sub>2</sub> species to phospholipids below their CMCs. With micelles of these substrate analogs clear differences were observed. Direct binding studies with the micellar substrate analog C16PN indeed show the great importance of a large hydrophobic residue at position 31. The bulky Trp residue improves the affinity of the enzyme for these micelles 4-fold, whereas the other five mutations cause a great reduction in affinity for C16PN micelles. However, with diC8diamidoPC as a micellar substrate analog, no such loss in affinity was observed with these five mutants. This interesting difference in affinity of these five mutants for the two micellar substrate analogs could be caused by several features of these compounds. The first difference is that C16PN has only one alkyl chain, whereas diC8diamidoPC contains two acyl chains resulting in a different mode of binding of the enzyme to these interfaces. For example, it could be that one acyl chain of diC8diamidoPC interacts with the enzyme, whereas the second acyl chain could serve as an 'anchor' when the enzyme is bound to these micelles. This effect could cancel out the effects of the mutations. The second difference between these two substrate analogs could be the way in which they are packed in a micelle. The CMC of C16PN is ~200 times lower than the CMC of diC8diamidoPC. It is generally accepted that micelles of compounds with a low CMC are more densely packed than micelles of compounds with a higher CMC. The interaction of pancreatic phospholipases is very sensitive to changes in packing density of aggregated phospholipids in monomolecular surface films and in bilayer systems (Verger and de Haas, 1976) and the interaction with micelles might be regulated in the same way.

If indeed a large hydrophobic residue on position 31 is important for the interaction with densely packed interfaces, one can understand that the mutants with smaller side chains have reduced binding to the more densely packed micelles, whereas binding to more loosely packed micelles remains unchanged. Our results show that the affinity of (mutant) enzyme for diacyl phospholipid micelles can be measured by direct binding studies with diacyl substrate analogs but that results obtained with monoalkylphosphocholines should be interpreted with care. The  $K_m$  values for the six mutants with diC8PC as a substrate agree quite well with the  $N \cdot K_d$  values derived from the direct binding studies with the non-hydrolyzable substrate analog diC8diamidoPC.

All mutants have reduced hydrolysis rates and the question arises what causes this effect. Several factors could contribute to this reduced catalytic efficiency. One of these factors could be that the three-dimensional structures of the mutants have been changed. We assume that the conformations of the mutants have not been altered significantly, at least not in the region of the active site, for the following reasons. The three-dimensional structures of bovine and porcine PLA<sub>2</sub> (Leu31) and of *C. atrox* PLA<sub>2</sub> (Trp31) are highly similar (Renetseder *et al.*, 1985). Moreover, the high variability in natural phospholipases for the residue at position 31, taken together with its location at the surface of the enzyme, does not suggest a structural role for this residue. However, in the case of Gly31, a small conformational change cannot be excluded *a priori*, in view of the known deviations in  $\phi$  and  $\psi$  torsion angles, which could influence the local conformation of the peptide chain. Indeed, this mutant displays the lowest activities and the lowest substrate affinities of all tested mutants. In 1985 van Scharrenburg *et al.* showed with laser-induced Eu<sup>3+</sup> luminescence studies that, upon binding of PLA<sub>2</sub> to monomeric substrate analogs, one water molecule was excluded from the active site. In the presence of micellar concentrations of this analog four water molecules were excluded and the authors proposed that this increased dehydration could be an important reason for the enhanced activity of PLA<sub>2</sub> at lipid–water interfaces. Our kinetic studies with the monomeric substrate diC6dithioPC showed that  $k_{\text{cat}}$  values were lowered by the mutations at 31. We assume that the introduction of a smaller and/or more polar residue than Leu at position 31 causes a less effective shielding of the active site by the residue at position 31, resulting in a less efficient dehydration process of the active site. With monomeric substrate the reduction in  $k_{\text{cat}}$  values is only moderate, whereas the reduction in hydrolysis rates is ~10-fold stronger with micellar substrates. This suggests that dehydration of the active site is more critically dependent upon the side chain at 31, when the enzyme is bound to interfaces rather than acting in water. Considering the mutations at 31 that affect activity, both the size and polarity of the amino acid side chains of these mutants could be important. The fact that the charged, but relatively large, arginine residue displays the highest catalytic rates of all polar residues at position 31 suggests that size could be more important than charge. To obtain a more complete insight in the role of size and polarity on catalytic rates and substrate binding it would be interesting to investigate the catalytic properties of mutants carrying any of the other 13 possible residues at position 31. We have restricted ourselves, however, to those residues that are encountered also in naturally occurring PLA<sub>2</sub>s, to ensure a high probability of correctly folded and active mutant enzymes. In addition to the above explanation for the decrease in activity of all the mutants, several other factors may play a role. First, it is conceivable that the size of the side

chain at 31, which is close to the entrance of the active site (Figure 1), influences the orientation of the substrate molecule relative to the active site residues. Second, it could also be that Leu31 gives a more favorable interaction with the transition state than the other six residues at position 31, resulting in lower  $k_{\text{cat}}$  values for the mutants. Third, although the dissociation constants ( $N\cdot K_d$  values) are known, the association and dissociation rate constants which determine this  $N\cdot K_d$  value are not known. Therefore, it is possible that the lifetime of the enzyme-micelle complex is different for all mutants. Because the complex contains a large number of substrate molecules per enzyme molecule and because the turnover numbers are low, variations in the life time of the complex may very well influence  $V_{\text{max}}$  values.

In 1976, Viljoen *et al.* found a large decrease in enzymatic activity of a PLA<sub>2</sub> from *B. gabonica* venom in which the Trp at position 31 was modified by *N*-bromosuccinimide. Because the enzyme was protected against this inactivation by micellar concentrations of substrate analogs these authors suggested that Trp31 in this PLA<sub>2</sub> was involved in substrate binding. Thus, inability of binding of the modified protein to substrate micelles was held responsible for the low catalytic rates of this enzyme. Our results show that the introduction of more polar residues at 31 reduces  $V_{\text{max}}$  rather than substrate binding. Because the oxidation of Trp31 in the venom PLA<sub>2</sub> creates a more polar side chain, the loss in activity after this chemical modification might very well be explained as a  $V_{\text{max}}$  effect.

In general, snake venom PLA<sub>2</sub>s, in which a Trp, Arg or Ala residue is frequently encountered at position 31, are very active on short chain lecithins. The enzymes from *Crotalus adamateus* and from *Naja melanoleuca* venom, which contain a Trp and an Arg residue at 31 respectively, are 10–20 times more active on diC8PC than porcine pancreatic PLA<sub>2</sub> (Wells, 1972; van Eijk *et al.*, 1983). Because introduction of either Trp or Arg in porcine pancreatic PLA<sub>2</sub> reduces rather than improves enzymatic activity, the enhanced activity of the venom PLA<sub>2</sub>s cannot be explained by the character of the residues at 31 only. Since the homology of pancreatic PLA<sub>2</sub>s with the sequences of snake venom PLA<sub>2</sub>s is ranging between 30 and 60% (Waite, 1987), changes in other parts of the sequence probably are responsible for the difference in activities. One such change is the surface loop between residues 62 and 66 which is present in pancreatic phospholipases but lacking in venom phospholipases. Indeed, removal of this loop from porcine pancreatic PLA<sub>2</sub> increases enzymatic activity on short chain lecithins considerably (Kuipers *et al.*, 1989b).

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